Fertility Analysis of Bovine Semen by in Vitro Fertilization

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Abstract

The aim of the present study was to evaluate the efficiency of using *in vitro* fertilization to validate semen fertility for artificial insemination. Cryopreserved semen from ten bulls (five Nelore and five Brangus bulls) was evaluated using *in vitro* production of embryos (IVPE) and via fixed-time artificial insemination (FTAI). There was variation ($p < 0.05$) in the IVPE (20.9 to 53.7% of blastocyst production) and in the FTAI (42.0 to 56.0% of pregnant cows) results among the Nelore bulls evaluated. According to the results there was a positive correlation ($rs = 0.8378$; $p = 0.0001$) between the rate of blastocyst production (using IVPE) and the rate of pregnancy (using FTAI) using Nelore bull semen. Variation was also found between the Brangus bulls ($p < 0.05$), in the rates of blastocyst production (36.5 to 47.0%) and pregnancy (45.6 to 52.2%) via FTAI. There was also a positive correlation ($rs = 0.8786$; $p = 0.0001$) between the rates of blastocyst production (IVPE) and pregnancy (FTAI) when using Brangus bull semen. According to the results, IVPE may be used in addition to conventional semen analysis to evaluate and validate the semen fertility of bulls for artificial insemination programs.

1. Introduction

Biotechniques such as artificial insemination or *in vitro* fertilization are commonly used in cattle to promote genetic improvement of the herd (Pellegrino et al. 2016). The semen from the bulls used for this purpose is mostly cryopreserved and purchased from Artificial Insemination Centers. However, the fertilization potential of this semen is highly variable due to the limitations of predicting the actual fertility status. This variation directly affects the herd's productivity.

The ability of a sperm to fertilize an egg *in vivo* is acquired as the gamete passes through the female's reproductive tract via a process known as "capacitation" (Binelli et al. 2018, Jin and Yang 2017). *In vitro*, sperm capacitation is acquired through the use of drugs (Jin and Yang 2017, Parrish 2014, Samardzija et al. 2006). Regardless of whether it is *in vivo* or *in vitro*, the ability and fertility potential of the semen is fundamentally important for the advancement of breeding biotechniques (Garcia-Alvarez et al. 2009). However, the evaluation procedures used in Artificial Insemination Centers essentially consist of a subjective diagnosis of the motility, concentration, and morphology of the semen, and of the integrity of the plasma and acrosomal membranes (Dias et al. 2009, Khalil et al. 2019). However, several other evaluations are also used, including the computerized sperm movement assessment procedure, morphofunctional analysis by fluorescent probes, flow cytometry, incubation testing, and sperm separation (de Arruda et al. 2015, Dias, Maciel, de Paula, Junior and Ozanan 2009, Khalil, El-Harairy, Zeidan and Hassan 2019, Rodriguez-Martinez 2007). Although Artificial Insemination Centers employ various evaluations that are indispensable and even irreplaceable (de Arruda, Celeghini, Garcia, dos Santos, Leite, Oliveira, Lançoni and de Paula Rodrigues 2015), there is a deficiency in our ability to predict the fertilization result of a sample from a given bull that is used for artificial insemination or *in vitro* fertilization. This uncertainty leads to concerns about the reliability of semen fertility and its correlation with field fertility.

A more reliable method for testing the fertility of a semen sample may be artificial insemination itself. However, artificial insemination can be time consuming and costly. *In vitro* embryo production could provide a viable and promising alternative. Using this tool it is possible to perform several simultaneous evaluations of different bulls, which would reduce the time required to validate semen fertility and possibly reduce costs. The aim of the present study was to evaluate the efficiency of *in vitro* fertilization as a method for validating semen fertility for use in artificial insemination.

2. Materials And Methods

2.1. Oocyte Selection

Chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise specified. For *in vitro* embryo production, the ovaries of cows were collected from the slaughterhouse and transported in NaCl solution (0.9%) plus 50 µg/mL gentamicin at a temperature of approximately 30ºC. Ovaries containing follicles with diameters of 3–8 mm were aspirated using a needle and syringe (Fidelis et al. 2020). Oocyte categorization was performed in a petri dish (Corning Incorporated, USA, 430487) under a stereomicroscope (Nikon, SMZ1000). Oocytes with more than two cumulus cell layers and a uniform cytoplasm were selected (de Almeida Barros et al. 2019).

2.2. *In vitro* Maturation

The selected oocytes were transferred to TCM-199 (M4530) maturation medium supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin, 0.2 µM pyruvate, 100 µM cysteamine and hormones (5.0 µg/mL Lutropin and 0.5 µg/mL Folltropin; Bioniche Animal Health USA, Inc.). *In vitro* maturation was performed in a Petri dish (Corning, 430166) in 100 µL drops of maturation medium (with 25 oocytes per drop), which were covered with mineral oil, for a period of 22 hours in a cell culture incubator at 38.5°C, with an atmosphere containing 5% CO_2 (Sovernigo et al. 2017) .

2.3. *In vitro* Fertilization and Culture

Cryopreserved semen from five Nelore bulls (N1–5) and five Brangus bulls (3/4 Red Angus and 1/4 Brahman; B1–5) was thawed at 36°C in a water bath for one minute and prepared according to the BoviPure density gradient technique (Nidacon International AB, Sweden). Oocytes and sperm (2×10^6 cells/mL) (Adona et al. 2020) were co-incubated in 100 µL droplets of fertilization medium (Parrish 2014) covered in mineral oil (Corning, 430166) for a period of 18 hours.

After *in vitro* fertilization, the oocytes were partially stripped with the aid of an automatic peptide and transferred to the *in vitro* culture medium (Holm et al. 1999, Sovernigo, Adona, Monzani, Guemra, Barros, Lopes and Leal 2017). *In vitro* cultivation was performed at 38.5°C in an atmosphere containing 5% CO2. The embryo (blastocyst) production rate was evaluated under a stereomicroscope (Nikon, SMZ1000) on the seventh day of cultivation.

2.4. Fixed-Time Artificial Insemination

The fixed-time artificial insemination (FTAI) procedures were performed during the reproductive season in a property located in the city Aragarças, Goiás State (GO), Brazil (15°53'51" S, 52°15'03" W). The climate is Aw according to the Köppen classification, with an average annual temperature of 25.7ºC and precipitation of 1,579 mm.

The cows were separated according to breed (Nelore [Zebuino] and Brangus [3/4 Red Angus and 1/4 Brahman]). The cows had body condition scores higher than 2.5 (1–5 scale) and were kept on a *Brachiaria brizantha* pasture, with access to water and mineral salt *ad libitum*. Sanitary control was performed according to the technical manual of the Ministry of Agriculture, Livestock and Food Supply (BRASIL 2009).

For the FTAI, the cows (Nelore and Brangus) were synchronized at a random estrous cycle stage using an intravaginal progesterone device (Sincrogest, Ourofino Animal Health, Brazil) and the intramuscular application of 2 mg estradiol benzoate (Estrogin, Pfizer Animal Health, Brazil) on Day 0. On Day 8, the intravaginal device was removed, and 150 g d-cloprostenol (Preloban, Intervet, Brazil), 300 IU equine chorionic gonadotropin (eCG; Novormon, Syntex, Argentina), and 1 mg estradiol cypionate (ECP, Pfizer, Brazil) was administered intramuscularly. The artificial insemination (single dose) was performed 48 hours later. The semen of five Nelore (N) and five Brangus (B) bulls was purchased from registered Artificial Insemination Centers. Thirty days after artificial insemination, a pregnancy diagnosis was performed by transrectal ultrasound using a DP-2200Vet ultrasound coupled to a Linear Transducer (Mindray of Brazil, Trade and Distribution of Medical Equipment Ltd.).

2.5. Statistical Analysis

Statistical analyses were performed using the Shapiro-Wilks test followed by the Kruskal-Wallis test to compare the rates of blastocyst production (via *in vitro* production of embryos [IVPE]) and pregnancy (via fixed-time artificial insemination [FTAI]). The Spearman's rank correlation test (rs) was used to verify the degree of association between the variables IVPE (blastocysts) and FTAI (pregnancy). *P*-values lower than 0.05 were considered significant.

3. Results

There were no significant differences ($P > 0.05$) in the rates of blastocyst production (IVPE) using the semen of Nelore (N) bulls N1 and N2. However, the rate of blastocyst production of bull N1 semen was superior ($P < 0.05$) to that of bulls N3, N4, and N5. Bull N2 was similar to bull N3 ($P > 0.05$), and bull N3 was similar to bull N4 ($P > 0.05$) in the rate of blastocyst production. However, the IVPE of bull N4 was significantly different ($P < 0.05$) from that of bulls N1, N2, and N5. Bull N5 had the lowest ($P < 0.05$) blastocyst production rate compared to the other bulls (Table 1).

Table 1
Evaluation of *in vitro* production of embryos (IVPE) and fixed-time artificial insemination (FTAI) using the semen of five Nelore bulls (N1–5).

IVPE	Oocytes	Blastocysts*	FTAI	Cows	Pregnancy**
	N	N (% ± SD)		N	N (% ± SD)
semen N1	149	80 (53.7 ± 2.0)[a]	semen N1	349	180 (51.6 ± 3.9)[ab]
semen N2	150	77 (51.3 ± 3.0)[ab]	semen N2	341	191 (56.0 ± 5.1)[a]
semen N3	150	70 (46.7 ± 2.1)[bc]	semen N3	214	108 (50.5 ± 6.3)[ab]
semen N4	150	68 (45.3 ± 2.1)[c]	semen N4	321	155 (48.3 ± 4.2)[bc]
semen N5	148	31 (20.9 ± 3.2)[d]	semen N5	131	55 (42.0 ± 5.5)[c]

Different letters in the same column (a–d) indicate a significant difference ($P < 0.05$) between bulls. The percentage (%) and the standard deviation of the mean (± SD) are based on six experimental repeats. *Seven days post *in vitro* fertilization - *in vitro* production of embryos (IVPE). **Pregnancy diagnosis after 30 days post fixed-time artificial insemination (FTAI).

The pregnancy rate (FTAI) using the Nelore bull semen did not differ significantly ($P > 0.05$) between the bulls N1, N2, and N3. The pregnancy rate of bulls N1 and N3 were also similar to that of bull N4 ($P > 0.05$), which was similar to that of bull N5 ($P > 0.05$). The pregnancy rate of bull N5 was significantly different ($P < 0.05$) to that of the bulls N1, N2, and N3 (Table 1). There was a positive correlation (rs = 0.8378; $P = 0.0001$) between the blastocyst production rates (IVPE) and the pregnancy rates (FTAI) using Nelore bull semen (Fig. 1A).

With regard to the Brangus (B) bulls, the rate of blastocyst production did not differ significantly ($P > 0.05$) between bulls B1, B2, B3, and B4 (Table 2). However, bull B5 had the lowest ($P < 0.05$) production of blastocysts compared to the other bulls evaluated (B1, B2, B3, and B4).

Table 2
Evaluation of *in vitro* production of embryos (IVPE) and fixed-time artificial insemination (FTAI) using the semen of five Brangus bulls (B1–5).

IVPE	Oocytes	Blastocysts*	FTAI	Cows	Pregnancy**
	N	N (% ± SD)		N	N (% ± SD)
semen B1	149	70 (47.0 ± 2.4)[a]	semen B1	299	156 (52.2 ± 6.2)[a]
semen B2	147	68 (46.3 ± 5.2)[a]	semen B2	304	149 (49.0 ± 6.2)[ab]
semen B3	150	67 (44.7 ± 3.9)[a]	semen B3	222	108 (48.6 ± 4.2)[ab]
semen B4	149	66 (44.3 ± 3.2)[a]	semen B4	270	133 (49.3 ± 5.1)[ab]
semen B5	148	54 (36.5 ± 3.5)[b]	semen B5	226	103 (45.6 ± 5.0)[b]

Different letters in the same column (a–b) indicate a significant difference ($P < 0.05$) between bulls. The percentage (%) and the standard deviation of the mean (± SD) are based on six experimental repeats. *Seven days post *in vitro* fertilization - *in vitro* production of embryos (IVPE). **Pregnancy diagnosis after 30 days post fixed-time artificial insemination (FTAI).

The pregnancy rate (FTAI) significantly differed between bulls B1 and B5 ($P < 0.05$). However the pregnancy rate of both bulls (B1 and B5) did not significantly differ ($P > 0.05$) from that of the other bulls evaluated (B2, B3, and B4). The pregnancy rates of the bulls B2, B3, and B4 did not significantly differ ($P > 0.05$) from each other (Table 2). There was a positive correlation (rs = 0.8786; $P = 0.0001$) between the IVPE and FTAI results using Brangus bull semen (Fig. 1B).

4. Discussion

Knowledge of the ability or fertility potential of semen is of fundamental importance for the advancement of breeding biotechniques (Garcia-Alvarez, Maroto-Morales, Martinez-Pastor, Fernandez-Santos, Esteso, Perez-Guzman and Soler 2009). The evaluations performed in this study found significant variation in fertility between bulls (semen) regardless of the breed (Nelore or Brangus) or the method used (IVPE or FTAI). This variability in semen fecundity is inherent in cattle and other species, and has already been described in the literature (Ferreira et al. 2017, Takeda et al. 2019, Tsakmakidis et al. 2010, Ugur et al. 2019). Bulls and their semen undergo various evaluation processes (for motility, viability, membrane integrity, morphology, capacitation, and acrosome reaction for example) before being marketed in specialized Artificial Insemination Centers (Dias, Maciel, de Paula, Junior and Ozanan 2009, Freitas et al. 2009, Maziero et al. 2009). Therefore, the variability in semen fecundity between bulls selected by the Artificial Insemination Centers should be able to be estimated prior to the application of the semen in the field. However, the results of such evaluations do not always correlate with semen fecundity results using IVPE or artificial insemination programs (Kumaresan et al. 2017, Morrell et al. 2018, Sudano et al. 2011). It is of fundamental importance that the fertility of bull semen that is intended for artificial insemination or IVPE programs can be better predicted using more reliable tests such as those performed in this study. These tests should be implemented in addition to the main evaluation methods that are already

employed at the Artificial Insemination Centers (Kumaresan, Johannisson, Al-Essawe and Morrell 2017, Morrell, Valeanu, Lundeheim and Johannisson 2018, Sudano, Crespilho, Fernandes, Junior, Papa, Rodrigues, Machado and Landim-Alvarenga Fda 2011).

The tests that are currently used in Artificial Insemination Centers present certain difficulties with regards to certifying semen fertility. This is because sperm are complex cells that need various attributes to perform their natural function in fertilization (Arruda et al. 2015, Maziero, Crespilho, Freitas-DellAqua, Junior, Antônio and Papa 2009). These inaccuracies in traditional semen evaluation procedures can generate significant variation in the fertility rates achieved in artificial insemination and in IVPE programs in cattle. This leads to losses that could be attenuated (Arruda, Celeghini, Garcia, Santos, Leite, Oliveira, Lançoni and Rodrigues 2015, Maziero, Crespilho, Freitas-DellAqua, Junior, Antônio and Papa 2009, Thundathil et al. 2016).

In recent decades, several laboratory methods have been developed in order to provide more accurate analyses of semen composition and structural integrity, with the aim of improving field performance (Arruda, Celeghini, Garcia, Santos, Leite, Oliveira, Lançoni and Rodrigues 2015, Khalil, El-Harairy, Zeidan and Hassan 2019, Kipper et al. 2017, Maziero, Crespilho, Freitas-DellAqua, Junior, Antônio and Papa 2009). These methods are indispensable but do not yet effectively predict semen fecundity. However, the association of these methods with *in vitro* embryo production may be essential for providing an estimate of the pregnancy rate for each semen batch or for each bull. This is supported by the results of this study. The rate of blastocyst production (IVPE) and pregnancy (FTAI) varied among the bulls in this study. However, most bulls had a good fertility performance, with values in line with those found in the literature and foreseen for these biotechnologies (Crites et al. 2018, Cunha et al. 2019, Franco et al. 2018, Sovernigo, Adona, Monzani, Guemra, Barros, Lopes and Leal 2017). The results of this study corroborate with the tests performed to evaluate semen in the Artificial Insemination Centers. This indicates that the evaluations performed in the Insemination Centers are indispensable, but more tests are essential for predicting semen fertility. These additional tests could include the IVPE performed in this study. The use of these additional tests could increase the reproductive efficiency of cattle and reduce the costs involved with management and resynchronization.

Comparing the rate of blastocyst production (IVPE) with pregnancy (FTAI) for both breeds of bull (Nelore or Brangus) evaluated in this study, there was a significant association between the variables. This indicates that increases in the rate of blastocyst production via IVPE for a given bull, correlates with increases in the rate of pregnancy via FTAI. The results of this study showed that IVPE can be used as an efficient tool to evaluate bull semen fecundity after tests carried out by the specialized Artificial Insemination Centers. The IVPE technique could be used to provide an estimate of the pregnancy rates for each bull. This could be used to validate semen fertility in preparation for artificial insemination and other assisted reproduction biotechnologies.

Despite the small number of bulls evaluated, the results of the present study indicate that IVPE may be used in addition to conventional semen analyses, to evaluate and validate the semen fertility of bulls

from Artificial Insemination Centers. Such a method could benefit biotechnological productivity by promoting predictability and increasing pregnancy rates through artificial insemination.

Declarations

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Conflicts of interest:

The authors declare that they have no conflict of interest.

Availability of data and material:

All data generated or analysed during this study are included in this published article

Code availability:

Not applicable

Authors' contributions:

All authors participated in the experimental design, and the laboratory and field evaluations. The authors also assisted in the preparation of the manuscript, read the final version, and agreed to submit the manuscript for publication.

Ethics approval:

The experimental design involving animals were in accordance with the ethical standards Pitágoras Unopar University ethics committee under the protocol 007/20.

Consent to participate:

Not applicable

Consent for publication:

Not applicable

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Figures

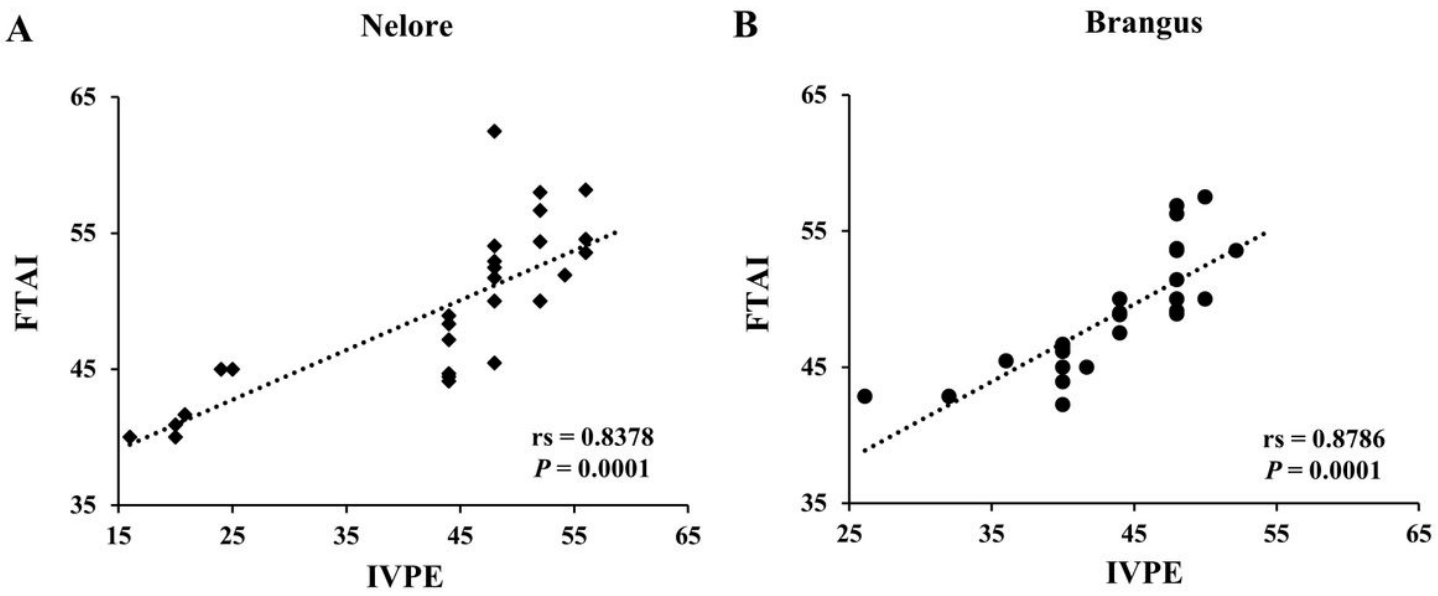

Figure 1

Degree of association between in vitro production of embryos (IVPE) and pregnancy by fixed-time artificial insemination (FTAI) using A) Nelore and B) Brangus bull semen. The Spearman's correlation test (rs) was used to assess the correlation.